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VARIABILITY AMONG ISOLATES OF
COLLETOTRICHUM GRAMINICOLUM
FROM SOME GRASSES IN ALBERTA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE

BY

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Variability among isolates of Colletotrichum graminicolum from some grasses in Alberta", submitted by Shook-Leng Lam in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Isolates of Colletotrichum graminicolum from six grass hosts differed considerably in some of their physiologic and morphologic characteristics. Host specialization, as shown within the limits of this investigation, occurred in the brome isolate only. Most other isolates attacked all hosts but with varying degrees of virulence.

Perhaps the greatest variability among isolates occurred in their rate of growth on different sources of nitrogen. Ammonium nitrate supported the best growth but the dry weight, measured after a prescribed period of time, varied considerably among isolates. Least growth occurred on ammonium sulfate and a moderate amount on potassium nitrate. The organic source of nitrogen, asparagine, also produced large variations in the amount of growth by the cereal crop isolates.

Variations in growth rate as affected by temperature or pH of medium were less marked than those on nitrogen media.

All isolates utilized starch but three of them, reedgrass, wheat and barley, attained considerably larger weights than the remaining three. Xylose and arabinose were utilized weakly, while methyl cellulose did not support growth of any isolate.

There were no differences in the rates of germination of conidia from different isolates.

Similarly there were no significant differences in sizes of conidia from different isolates.

Two of the isolates, wheat and brome, were uninucleate, while the remaining isolates were multinucleate.

Differences in colony characteristics of the isolates were striking.

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INTRODUCTION

Anthracnose, caused by Colletotrichum graminicolum (Ces.) Wils., is distributed widely on cereals and grasses. Sprague (36) reports 113 species of grasses as hosts of this fungus in Canada and the United States. Its occurrence in Alberta was first reported in 1933 by Sanford (30), and has since been found on all our cereals and most of our grasses.

In 1963 Harder (18) made an extensive survey of this disease in Alberta and reported that oats is most frequently affected, although barley and wheat are sometimes severely attacked by this fungus. His investigation also showed that the main factors influencing the severity of anthracnose are: the low nitrogen requirements of C. graminicolum; the low temperature for optimum sporulation and high temperature for disease development; and the high sensitivity of this fungus to certain microbial antagonists.

Anthracnose of grasses is difficult to diagnose during the major period of plant growth. General reduction in vigor of plant development, weakened, thin stems, and premature ripening are common symptoms. These conditions can be easily mistaken for those caused by low or unbalanced soil fertility or by drought. Signs of the disease, consisting of black, setose acervuli, appear when the plant is mature. It is at this stage that the cause of the disease can be easily determined.

Symptoms of anthracnose are somewhat similar on most grasses, but even casual observation of the fungus suggests that differences occur between isolates from different hosts.

The pathogen, C. graminicolum, regardless of the host on which it appears may be described as follows: Acervuli dark brown or black, elongated; setae few or many, dark brown or black 1 - 2 septate 60 - 120 microns long and 6 - 8 microns thick at the base; conidiophores very short, 6 - 12 x 1 - 2 microns; conidia falcate, spindle- or boat-shaped, 2-several guttulate, 18 - 26 x 3 - 4 microns.

Dicladium graminicolum Ces. was the name first given to the causal organism of anthracnose of cereals and grasses by Cesati in 1852 (7). Manns, in the paper of 1909 by Selby and Manns (32), gave the name Colletotrichum cereale to the anthracnose fungus. This has been one of the more commonly used names to describe the fungus. Wilson (37) in 1914 made the combination and named it Colletotrichum graminicolum, and listed 10 synonyms. Böning and Wallner (8) have listed 8 other names they consider to be synonymous with C. graminicolum. Since 1914 the latter name has been used almost extensively. Arx (5) considered C. graminicolum to be the imperfect stage of Glomerella tucumanensis.

The purpose of this investigation was to determine the extent of physiologic and morphologic variability among

isolates from some grasses. The morphologic features included spore size, colony characteristics in artificial medium and nuclear phenomena. The physiologic properties studied included: factors affecting pathogenicity; host specialization; rate of growth as influenced by different temperatures and reaction of medium; and nutritional requirements involving some sources of nitrogen and carbon.

I. HOST SPECIALIZATION AND PATHOGENICITY

Literature Review

Since C. graminicolum parasitizes a large number of hosts, and causes somewhat similar symptoms, it is of interest to determine to what extent host specialization occurs. Extensive work on the problem of physiologic strains of the anthracnose fungus has been done by Bruehl and Dickson (9). They found that foliage inoculations demonstrated specific strains most readily. He obtained a marked degree of host specificity in the isolates from oats, rye, barley and several other grasses. When cereals and grasses were not predisposed by unfavorable environmental conditions, disease was produced only by the isolates from the same or closely related species.

Sanford (30) did not obtain any infection on wheat, barley or flax with an isolate from oats. However, Winter (7) stated that isolates from oats, wheat, barley or rye were pathogenic upon each of the other hosts.

Bell (7) reported good anthracnose development on seedlings of Thorne and Trumbull wheat, and on Balbo and Rosen rye by isolates from wheat, rye or Bromus secalinus, but not by isolates from Lolium multiflorum, Hedera helix, barley or oats. He also found that Thorne wheat was more resistant to the pathogenic isolates than was Trumbull wheat.

Ali (4), in his investigation on the pathogenicity of isolates of C. graminicolum from wheat, alfalfa and orchard grass on 6 varieties of wheat (Dual, Butler, Knox, Lucas, Seneca and Vermillion), found that only the wheat isolate was pathogenic on wheat. Of these varieties of wheat, Dual was highly susceptible and Knox variety was highly resistant. He also reported that the pathogenicity of the wheat isolate was influenced by certain inorganic compounds and the hydrogen ion concentration.

Specialized races that constitute a species of a pathogen may respond differently to any single or a combination of factors. Temperature relations of the host and parasite may play an important part in the rapidity and extent of disease development. Certain diseases involve only mature plants or plant parts, while others are limited to seedlings or to young tissues.

Anthrachnose of cereals and grasses is usually associated with host maturity. This association, as stated by Bruehl and Dickson (9), is caused, at least in part, by the influence of temperature upon the fungus. This group of hosts matured as summer and higher temperatures progressed. In contrast to this group of cold-tolerant hosts, the Sorghum spp. suffered severely at the height of their vegetative activity, and no association with maturation was noted in this group. The higher heat requirements of isolates from Sorghum spp. were interpreted as an adaptation of parasite to host.

Bruehl and Dickson (9) also reported that development of anthracnose in Sudan grass seedlings was not influenced appreciably by temperature, and seedlings were blighted at soil temperatures from 16° to 32° C. However, in rye low temperatures greatly reduced or prevented anthracnose development. Rye seedlings suffered little at soil temperatures of 8° to 12° C. At 16° C and above, seedling damage of rye became severe.

They further reported that the presence of soil microflora is influenced by both soil temperature and moisture, which in turn has an indirect effect on C. graminicolum and disease development. Thus, the overall effect of temperature was not significant in sterilized soil.

Materials and Methods

(a) Isolation and Hosts

C. graminicolum was isolated from the following hosts: oats (Avena sativa), barley (Hordeum vulgare), wheat (Triticum aestivum), wild oats (Avena fatua), brome (Bromus inermis) and reedgrass (Calamagrostis canadensis). The diseased plant material was collected within about a 50-mile radius of Edmonton, Alberta. No attempt was made to determine the varietal name of the cereals from which this fungus was isolated.

The following cereal varieties were used in pathogenicity studies: Victory oats, Parkland and Thatcher wheat.

The fungus was isolated from the acervuli appearing on the lower portions of stems of mature plants. Short sections

of stem bearing acervuli were surface-sterilized for 1 minute in 1:1000 mercuric chloride solution, washed for several minutes in distilled water, and transferred to water agar in Petri plates. About 5 pieces were spaced in a plate and incubated at 15 - 20° C for 4 to 5 days. Bits of mycelium from the advancing edge of the colony were aseptically transferred to potato-sucrose agar in test tubes. Since C. graminicolum grows out of infected material relatively rapidly the above method produced a high percentage of pure cultures.

As a further precaution to ensure purity of cultures each of the isolates was single-spored by the method described by Beadle and Tatum (6). Subcultures of these single-spore isolates were maintained on PSA and were used for all subsequent studies.

(b) Inoculation Technique

Several methods of artificial inoculation were compared. These techniques included: (1) spraying of leaves with a suspension of conidia and fine mycelium, (2) drenching the soil around the stem with a suspension described above, and (3) injecting a suspension of conidia and macerated mycelium into the first internode above the soil by means of a hypodermic needle and syringe.

In all instances the injection method proved to be the most reliable for initiating infection and was, therefore, used in all subsequent pathogenicity tests.

(c) Host Specialization Tests

Host specialization tests of the 6 isolates of C. graminicolum were conducted on potted plants in the Victory variety of oats, the Thatcher variety of wheat, the Parkland variety of barley and the Dakold variety of rye.

Beginning at the 2 - 3 leaf stage of development the plants were inoculated by the injection method. Altogether 5 inoculations were made at the intervals of 10 - 15 days. Each treatment included 5 plants per pot in 4 different pots. A control consisting of the same planting without the inoculation was designed to detect the possible presence of soil-borne inoculum.

Results of inoculations were recorded when the plants were fully matured and dried. Infection was determined by the presence of elliptical, black, setose acervuli located on the lower portion of the culm. No attempt was made to record the severity of the disease.

(d) Pathogenicity Tests

(i) Effect of age of plant upon infection

Similar inoculations were performed at various stages of plant growth from young seedlings to maturity. The barley isolate was used on barley in this test since this combination gave the highest infection results when compared to any other isolate-host combination attempted.

A set of 10 pots, each containing 5 barley plants, was seeded at 5 different times 10 - 12 days apart. When the last set of plants was at a 2 - 3 leaf stage, inoculum of the barley isolate was injected into the culm of the lower internode of all plants in this experiment. Only one inoculation was performed. The plants were rated for infection at maturity.

(ii) Effect of soil temperature on infection

The effect of soil temperature on anthracnose development was conducted in Wisconsin soil - temperature tanks. One hundred and twenty-five barley plants were grown in 16 pots at a soil temperature of 7° C, and a similar number was grown at 24° C.

The plants were inoculated by the injection method 4 different times at 10 - 12 day intervals. The first inoculation was made at the 4-leaf stage. The plants were rated for disease development at maturity.

Results

(a) Host specialization

The pathogenicity of the 5 grass isolates of the anthracnose fungus varied. The one from brome produced no infection, while the one from barley gave the highest level of infection (Table I).

Table I. Pathogenicity of isolates of Colletotrichum
graminicolum from 5 different grasses on 4 cereal crops

Host	Percentage of plants infected by isolates from:				
	Barley	Wheat	Oats	Reedgrass	Brome
Barley	97	80	20	15	0
Wheat	35	15	0	10	0
Oats	91	96	84	15	0
Rye	99	45	10	14	0

The isolate from reedgrass was weakly pathogenic on all the cereals tested. The oat isolate displayed a somewhat similar effect except for the high percentage of infection on the crop from which it was isolated. The infective ability of the wheat isolate was somewhat erratic in its pathogenicity on the 4 cereals. It was highly pathogenic on barley and oats, moderately pathogenic on rye, and weakly pathogenic on wheat. The barley isolate showed the highest consistent degree of pathogenicity on all cereals except wheat where it was only moderately pathogenic.

Wheat showed the highest degree of resistance to all isolates.

(b) Effect of Age of Plant on Infection

There was some indication that within the age limits of 15 to 69 days, the older the barley plant, the greater the possibility of infection by the anthracnose fungus. The

percentage of plants infected (in brackets), depending on their age in days at the time of inoculation was as follows: 15(0); 28(50); 39(46); 47(47); 57(51) and 69(73).

Infection did not occur in 15-day old plants, and reached its highest level in 69-day old plants. There did not appear to be any marked difference in the percentage of plants infected between ages of 28 to 57 days.

(c) Effect of Soil Temperature on Infection

In this test 125 plants matured in soils that were constantly maintained at 7° and 24° C respectively. There were 73 percent of plants infected at 7° C and 79 percent infected at 24° C. Soil temperature, at least at these 2 levels, does not appear to influence the amount of infection of barley by C. graminicolum.

Discussion

The isolate of C. graminicolum from brome grass was the only one that showed evidence for strict host specificity within the limits of the grasses tested in this investigation. The isolates from barley, wheat and reedgrass infected the 4 cereal hosts in various amounts, while the one from oats infected all cereals except wheat. This reaction of the oat isolate is partly in agreement with that reported by Sanford (30). In these tests the oat isolate did not infect wheat but contrary to Sanford's results it did attack barley.

Wheat showed the highest degree of resistance to all isolates. It is particularly difficult to understand why this resistance was so high to the isolate originating from this same crop. It is possible that the method of inoculation used, or the age of plant at the time of inoculation could be factors in its resistance. Furthermore, the variety, Thatcher, used in this investigation may show a greater degree of inherent resistance. Different degrees of resistance in wheat varieties were demonstrated by Bell (7) and Ali (4).

Barley was highly susceptible to the barley and wheat isolates, while oats was highly susceptible to the 3 cereal isolates. Harder (18) reported that, in most areas of Alberta, oats and barley suffered most severely from anthracnose. The above results may explain the reasons for this situation. The 3 cereals, wheat, oats and barley are commonly used in rotation in Alberta. This type of rotation would ensure a high build-up of inoculum which is highly pathogenic to barley and oats.

The evidence for a positive correlation between age of barley plants and the numbers infected is fairly strong. Barley seedlings up to about the 3-leaf stage appear to be highly resistant. This lack of anthracnose development in the seedling stage of barley is not in agreement with the findings of Sanford (30) who considered seedling blight of oats to be quite prevalent in Alberta. However, this development of anthracnose occurred primarily on the roots of oats, whereas barley in these experiments was inoculated in the internode above ground.

Contrary to a report (9) that anthracnose isolates from grasses are usually favored by higher temperatures, there were no differences in the infection rate at 7 and 24° C. However, the reliability of the control of temperature immediately above the soil may be questioned. The air temperature in these experiments was about 18° C and, therefore, it no doubt modified the temperatures at soil level so that the temperatures at points of inoculation were not necessarily those of the soil. The effect of soil temperature on anthracnose development may operate indirectly through the extent of development of microbial antagonists. This effect would apply primarily to disease development in below ground plant parts. Bruehl and Dickson (9) obtained evidence that the over-all effect of temperature was not significant in sterilized soil.

II. NITROGEN AND CARBON UTILIZATION

Literature Review

(a) Nitrogen Requirements

Fungi can utilize inorganic or organic sources of nitrogen but they differ in the quality and quantity of these compounds that they require for optimum growth.

Among the inorganic sources, nitrates are considered by many workers, as reviewed by Cochrane (13), to be excellent for a large number of fungi. According to Robbins (28), any fungus that can utilize nitrate nitrogen can also utilize ammonium nitrogen. However, as shown in a review by Cochrane (13) the utilization of ammonium by a fungus often causes a sharp drop in the pH of the medium. This increase in acidity is apparently caused by a preferential utilization of the ammonium ion. Thus, the inability of a fungus to utilize ammonium nitrogen may reflect only its inability to grow at a low pH.

Kurtz and Fergus (20) reported that ammonium sulfate is a poor source of nitrogen for Colletotrichum coccodes because of the highly acid conditions that develop in this medium.

It has also been demonstrated that better growth may be obtained from organic than inorganic nitrogen. Hawker (19) stated that Phycomyces blakesleeana grew more rapidly on asparagine as compared to any inorganic source.

L (+) asparagine and yeast proved to be a good source of nitrogen for an alfalfa isolate of C. graminicolum, while L (-) arginine, ammonium nitrate or potassium nitrate yielded good growth for a wheat isolate of the same fungus (2).

Harder (18) found that an oat isolate of the anthracnose fungus had a relatively low requirement for inorganic nitrogen for optimum growth as compared to Rhizoctonia solani or Fusarium culmorum.

(b) Carbon Utilization

Lilly and Barnett (22, 23) in their work and review on the utilization of sugars by fungi reported that fungi vary in their ability to utilize different sugars for growth. Not only genera and species, but also isolates of fungus species have shown variation in their ability to utilize a given sugar. The response to various carbon sources may be specific, so that one substance may be used readily, while another of closely similar chemical structure may be unavailable.

Most fungi grow readily in sucrose and hexoses-glucose, mannose, fructose, and to a less degree in pentoses-xylose and arabinose. Margolin (24) observed that xylose was utilized by some fungi more readily than arabinose. Lilly and Barnett (23) confirmed this and reported that xylose was the better sugar for most fungi. Only a few species utilized arabinose better than xylose. Rama-Krishnan (27) found that Colletotrichum falcatum utilized arabinose and xylose poorly. These 2 sugars also proved to be unsuitable for the growth of C. gloeosporioides (37).

Starch is utilized by a number of fungi. Twenty six isolates and species of Saprolegniales were found to utilize starch readily (22). Failure of certain species to utilize various polysaccharides may be due to their inability to hydrolyze the sugars.

The ability to utilize cellulose as a carbon source has been shown in some fungi. Scales (31) studied the cellulose-decomposing ability of 30 species of Penicillium and 10 species of Aspergillus, the growth of which on plates was usually accompanied by the enzymic production of a cleared zone around the colonies. Cochrane (13), in a review, stated that many species of Ascomycetes and Fungi Imperfecti are able to decompose cellulose. The wood-rotting fungi of the Basidiomycetes are especially capable of decomposing cellulose. He also stated that the utilization of cellulose is affected by the available nitrogen, by temperature and other carbon compounds.

As shown in the previous section, the isolates of C. graminicolum differed in their pathogenicity on cereal hosts. Since pathogenicity may be associated with nutritional requirements of the pathogen it was desirable to compare the nitrogen requirements of these isolates from grass. In addition, the ability of these isolates to utilize such carbon sources as xylose, arabinose, starch and cellulose was determined. These nutritional requirements may thus provide further evidence of the variability that exists among the isolates of C. graminicolum.

Materials and Methods

(a) Nitrogen Requirements

Three inorganic compounds, NH_4NO_3 , KNO_3 and $(\text{NH}_4)_2\text{SO}_4$, and one organic compound, DL-asparagine were used as sources of nitrogen to determine whether the isolates of C. graminicolum showed any variability in their requirements for these elements.

A basic medium, described by Converse (14) to be the best carbon-salts combination for the growth of Helminthosporium gramineum was found suitable for the growth of C. graminicolum, and was used in all subsequent experiments on nutritional requirements.

The following medium excluding the nitrogen source, and designated for further reference as 'basal', was used:

Sucrose	38.0 g.
KH_2PO_4	0.3 g.
K_2HPO_4	1.2 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3 g.
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	1 ppm.
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1 ppm.
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1 ppm.
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	1 ppm.
Demineralized water .	1000 ml.

Four different media were prepared, each one consisting of the basal medium supplemented by each of the nitrogenous compounds. For each of the 3 inorganic sources of nitrogen

the amount of growth in each of the following concentrations of g nitrogen per litre was measured: 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6.

Growth in asparagine continued at considerably higher levels of nitrogen and therefore, the following concentrations per litre were used 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 8.0 g. The wheat, oat and barley isolates were checked on asparagine. The 200-ml Erlenmeyer flasks were washed in high detergent liquid, rinsed, and soaked overnight in distilled water, and rinsed twice again.

Fifty ml of medium was added to 200-ml Erlenmeyer flasks which were stoppered with cotton plugs and sterilized by autoclaving for 15 minutes at 15 lbs. steam pressure.

Inoculum was obtained by homogenizing 8-day old cultures of the fungus in sterilized distilled water in a Waring blender. Two ml of the suspension were pipetted into each flask. The material was incubated at 24° C for approximately 7 days. Each treatment was replicated 4 times.

Growth of the fungus was determined by measuring the dry weight of the mycelium following incubation. The fungus was filtered, rinsed several times with demineralized water and dried at 90° C for 24 hours in a forced-air oven. Filter papers were oven-dried at the same temperature and pre-weighed. The weights were recorded in mgs.

(b) Carbon Utilization

The purpose of this test was to determine whether the various isolates of C. graminicolum could utilize certain pentoses and polysaccharides. There was no attempt to determine the carbon concentration for optimum growth. The basal medium was supplemented with 0.15 g nitrogen per litre from KNO_3 . The cellulose medium was obtained by adding 12 g of methyl cellulose to the above medium. The xylose or arabinose medium was prepared by adding 36 g of d(+) xylose or 36 g of d(-) arabinose per litre of the above medium. Difco certified soluble starch was added to the basal medium with KNO_3 in the following manner: 1.5 g starch was placed in a sterile, stoppered flask and 5 ml of 95 percent ethyl alcohol were also added. The alcohol was allowed to evaporate at 25° C for several days in a forced-air oven. Fifty ml of the sterile basal medium containing KNO_3 was then added to the alcohol-sterilized starch.

Otherwise the procedures for incubation and measurement of growth were the same as those described under nitrogen nutrition.

Results

(a) Nitrogen Requirements

All isolates of C. graminicolum grew well in an ammonium nitrate medium, and although the optimum concentration of nitrogen varied mostly between 0.2 and 0.4 g per litre, there was a large difference in the maximum growth achieved by the different isolates (Fig. 1).

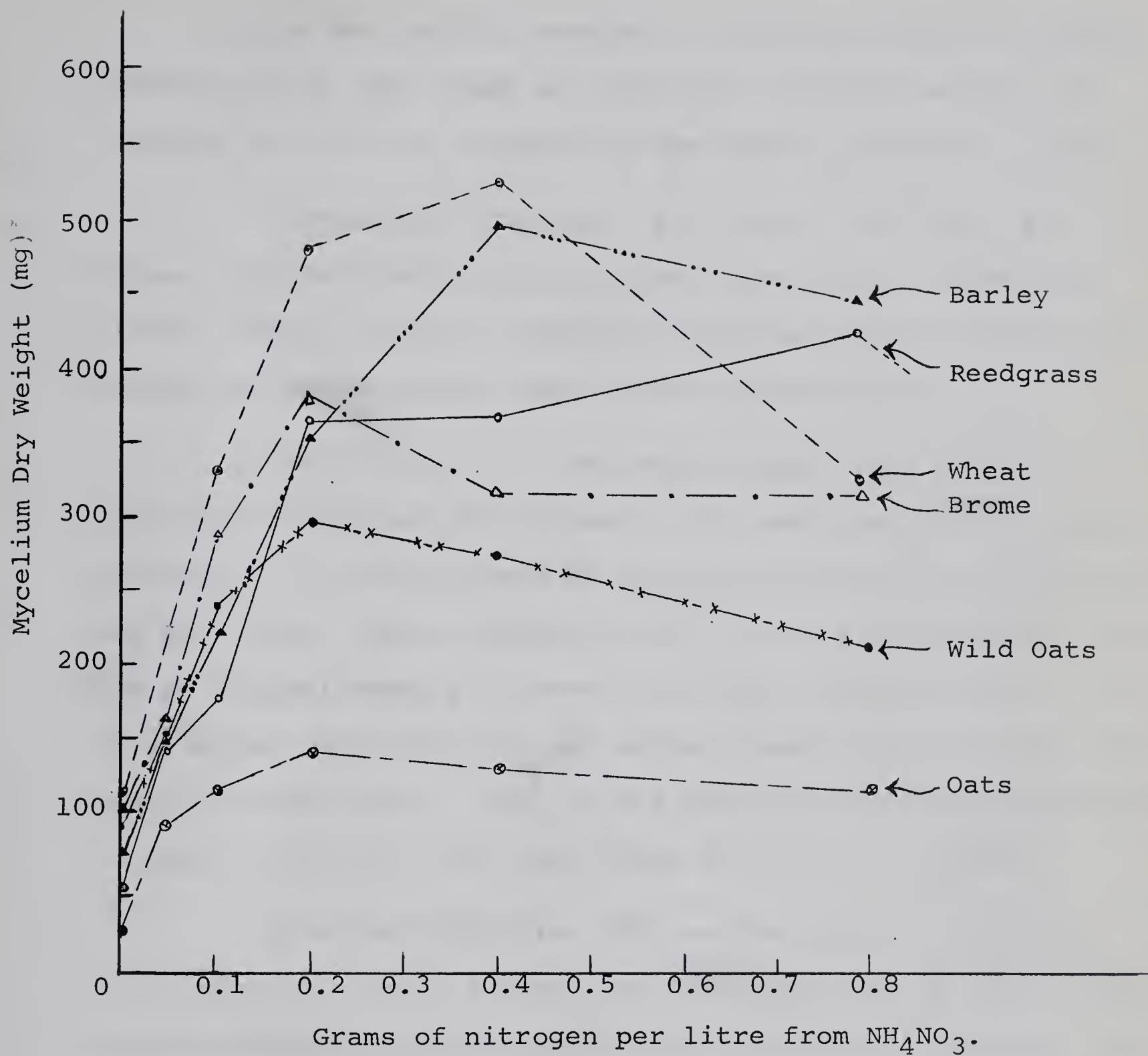


Fig. 1. Dry weights of mycelium of isolates of Colletotrichum graminicolum obtained in different concentrations of nitrogen from ammonium nitrate.

The oat isolate reached its maximum weight at approximately 135 mg per flask as compared to maximum weights of slightly over 500 mg reached by the wheat or barley isolate.

Three of the isolates, i.e., oats, wild oats and brome, required about 0.2 g nitrogen from NH_4NO_3 for maximum growth, while 3 others, reedgrass barley and wheat reached this maximum at approximately twice that concentration.

The initial pH of the media ranged from 6.8 to 7.2. Following the period of incubation the reaction remained fairly constant with little change at concentrations up to 0.1 g nitrogen per litre. Above concentrations of 0.1 g nitrogen per litre the pH dropped sharply in most instances, reaching a low of 2.1 in a medium where the wild oat isolate was grown at a 0.8 g per litre nitrogen level. Most of the other medium reactions ranged between 3.2 and 6.1 with most being in 4.8 to 5.8 range.

In media containing KNO_3 as the source of nitrogen there were also great differences among isolates in the maximum growth achieved, and the maximum concentration of nitrogen for optimum growth was approximately between 0.1 and 0.2 g nitrogen per litre of medium (Fig. 2).

The wild oat isolate produced the least amount of maximum growth of about 70 mg at approximately 0.2 g nitrogen per litre, while the barley isolate attained a weight about 340 mg at approximately the same concentration of nitrogen. The brome isolate attained a maximum weight somewhat similar to the barley one and at about the same nitrogen level. Oat

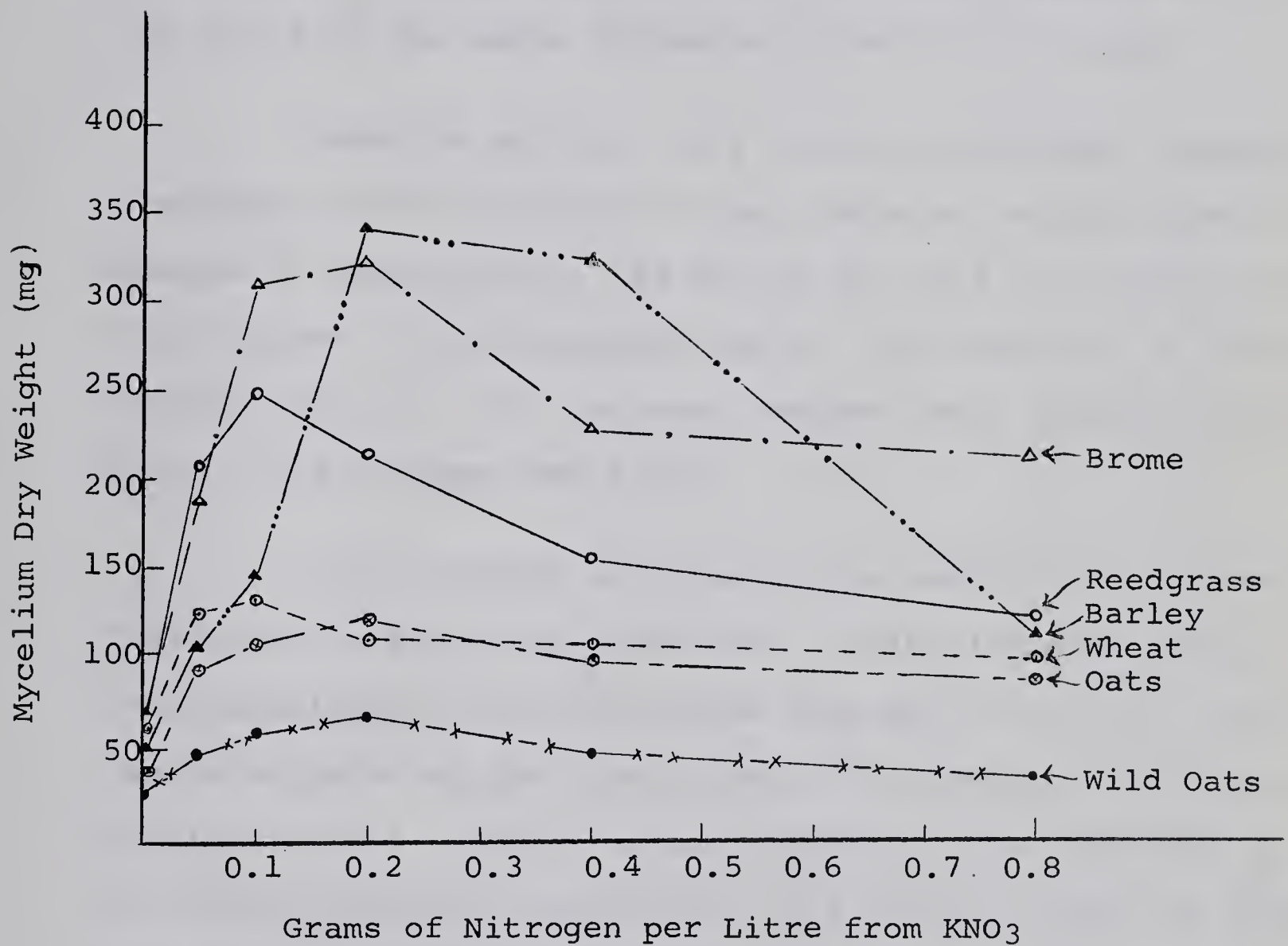


Fig. 2. Dry weight of mycelium of isolates of Colletotrichum graminicolum obtained in different concentrations of nitrogen from potassium nitrate.

and wheat isolates were relatively low, while the reedgrass isolate was intermediate in maximum growth attained.

The pH of the media did not change appreciably in any situation. It varied from 7.2 to 7.0 in the initial medium and 7.0 to 7.4 in the media following growth of the fungi.

Ammonium sulfate, as a source of nitrogen, supported a moderate amount of growth of all isolates varying from a low maximum of approximately 145 mg for the wild oat isolate to a high maximum of approximately 200 mg for reedgrass or brome isolate (Fig. 3). All isolates reached their maximum growth at about 0.1 g nitrogen per litre.

Large changes occurred in the reaction of the medium following the period of incubation. Initially media at all concentrations of nitrogen ranged from pH 6.5 to 7.0. Little change occurred at the lowest level of nitrogen, but at concentrations of 0.1 to 0.8 g nitrogen per litre the reaction of media following incubation ranged from pH 2.7 to 4.3 with the majority being close to pH 3.1.

The amount of growth of only 3 isolates of C. graminicolum, i.e., from wheat, oats and barley was determined on media with asparagine as the source of nitrogen.

The maximum amounts of growth attained by the 3 cereal isolates differed considerably with wheat attaining about 540, oats about 350, and barley about 140 mg of dry mycelium (Fig. 4).

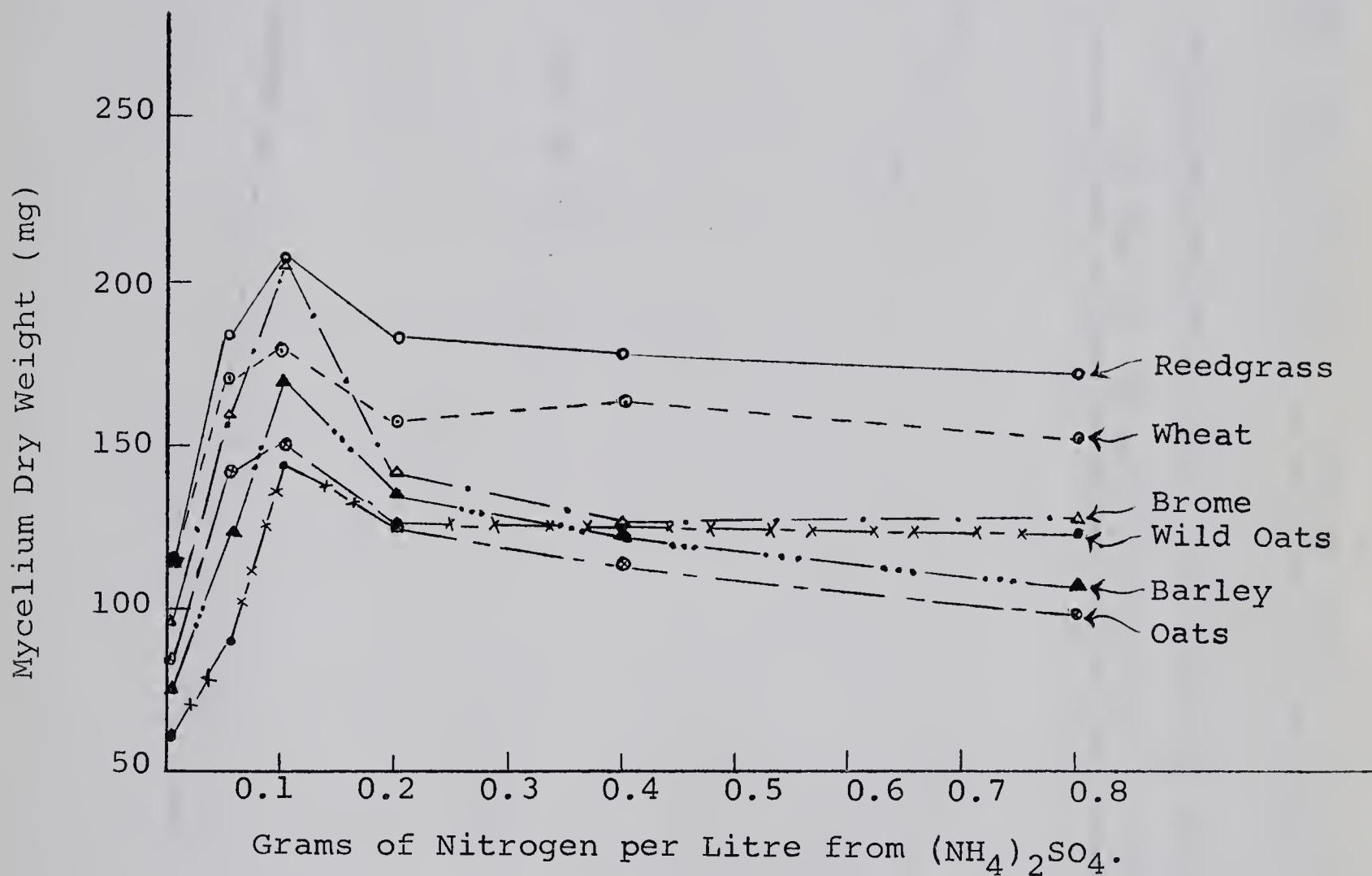


Fig. 3. Dry weights of mycelium of isolates of Colletotrichum graminicolum obtained in different concentrations of nitrogen from ammonium sulfate.

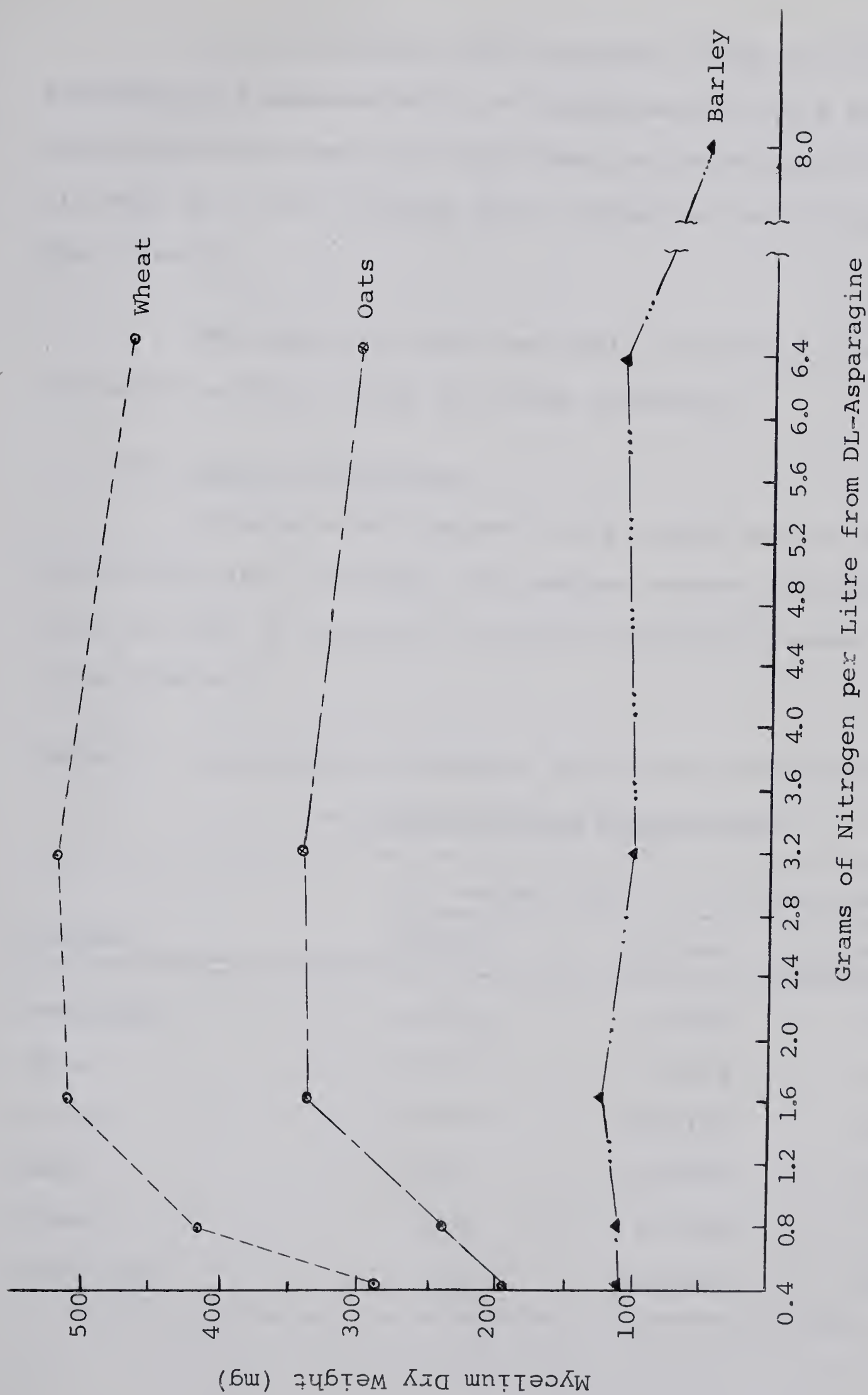


Fig. 4. Dry weights of mycelium of isolates of Colletotrichum graminicolum obtained in different concentrations of nitrogen from asparagine.

In all instances this maximum amount of growth was attained at a concentration of approximately 1.6 g nitrogen per litre and maintained this high level of growth up to about 6.4 g nitrogen per litre, beyond which concentration it dropped somewhat rapidly.

The rate of growth was rapid, attaining its maximum dry weight within 5 days following incubation.

(b) Carbon Utilization

Soluble starch proved to be a good source of carbon for all isolates, although the maximum amount of growth attained after 14 days of incubation varied considerably among some isolates (Table 2).

Table 2. Utilization of starch, xylose and arabinose by isolates of Colletotrichum graminicolum

Isolate	Dry weight (mg) of mycelium obtained on		
	Starch	Xylose	Arabinose
Reedgrass	0.5726	0.0496	0.0154
Wheat	0.5222	0.0839	0.0385
Barley	0.5176	0.1114	0.0255
Oats	0.2011	0.0624	0.1935
Brome	0.1934	0.0328	0.0095
Wild Oats	0.1148	0.0307	0.0094

Isolates from reedgrass, wheat and barley attained a level of growth approximately 3 times the amount produced by the oats, brome and wild oat isolates.

Xylose and arabinose were utilized weakly. In all instances, except the barley isolate, xylose appeared to be more readily available as a carbon source.

There was no growth of any isolate in media in which cellulose was the only source of carbon.

Discussion

Of the 3 inorganic sources of nitrogen, ammonium nitrate supported the best growth of all isolates of C. graminicolum. The maximum growth attained during the incubation period varied considerably among isolates grown on any nitrogen source. Both the ammonium and nitrate sources of nitrogen were utilized effectively. The reaction of the medium following incubation may not have played a major role in growth inhibition except perhaps at the upper levels of ammonium sulfate and in the highest concentration of ammonium nitrate.

The ability of all isolates to utilize nitrate and ammonium forms of nitrogen confirms the report of Robbins (28) that any fungus can utilize one form usually can utilize the other one as well. Ammonium sulfate is only a fair source of nitrogen for growth of most isolates. Kurtz and Fergus (20) came to the same conclusion regarding Colletotrichum coccodes.

The highly acid conditions that develop in the medium appear to be a major factor contributing to this weak growth.

Asparagine, as a source of organic nitrogen, was tested on only 3 isolates from cereals, viz., barley, wheat and oats. Here again, there was a great difference in the maximum growth attained by each of them. Barley utilized asparagine nitrogen poorly when compared to its utilization of inorganic nitrogen. Wheat and oats, however, utilized it as well or considerably better than some inorganic sources.

An interesting feature in the utilization of asparagine is that there was no peak with a fairly sharp drop in the weights attained even at high levels. Instead the maximum growth was reached at a concentration of about 1.6 g nitrogen per litre, and this approximate rate of growth was maintained till about the 6.4 g nitrogen per litre. The pH of the medium remained fairly constant throughout the entire range of concentrations. On this basis it may be argued that the 3 isolates received an optimum amount of nitrogen at 1.6 g per litre and that at higher concentrations there was a surplus of nitrogen but that this excess had no deleterious effect on the fungus.

In experiments of nitrogen requirements of the different isolates of C. graminicolum there was no attempt to define precisely the peak at which maximum growth was achieved. For this reason only a wide range of concentrations was used. This wide range served the purpose of detecting variability

among isolates in the utilization of nitrogen sources.

Insofar as utilization of carbon from different sources is concerned, all the isolates reacted similarly in relation to methyl cellulose. None of them could utilize this compound. The inability of C. graminicolum to utilize cellulose agrees with the review by Cochrane (13) that this property is common only in wood-destroying fungi.

Soluble starch was an excellent source of carbon for the reedgrass, wheat and barley isolates, but it was only moderately available to the remaining 3 isolates.

Both xylose and arabinose were utilized weakly by all isolates. Xylose was a better source of carbon for all isolates except the one from oats. Although these compounds were only slightly available to all isolates, there was considerable variability in utilization among isolates.

III. GROWTH AND REPRODUCTIVE CHARACTERISTICS

1. Effect of Temperature and of Reaction of Medium

Literature Review

Temperature is one of the important environmental factors affecting the metabolic activities of fungi. Wolf and Wolf (39) in their review of relations between fungi and temperature, stated that relatively few are active at 42° C and above, or 5° C and below. The optimum temperature for growth of most plant pathogenic fungi falls in the 20 - 30° C range.

Edgerton (15) reported optimum and maximum temperatures for Colletotrichum lagenarium to be 24 and 35° C, and for C. lindemuthianum to be 23 and 31° C respectively. Harder (18) found the optimum temperature for growth of the oat isolate of C. graminicolum to be between 28 and 30° C. Ali (3) found two temperatures, 20 and 30° C, that were optimum for the growth of isolates from wheat, alfalfa and orchard grass in yeast extract medium. Multiple optima were also reported by Mitchell and Houlahan (26) for the growth of Neurospora crassa at 23 and 37° C.

Fungi tolerate different ranges of pH of medium for their growth. Cochrane (13) stated that most plant pathogens grow best in media with initial pH of 5.0 to 6.5. Colletotrichum lindemuthianum is sensitive to acid media, and grows best under slightly alkaline conditions. Most species of Colletotrichum, however, are noted for their ability to grow over a wide pH range.

Small (35) reported that C. circinans grew in a pH range of 2.6 to 8.0. Chowdhury (12) found the optimum pH for growth and sporulation of C. capsici to be between 5.0 and 6.0. Agnihotri (1) confirmed Chowdhury's findings that best growth occurred at pH 6, but that it was able to grow in a range of pH 2 to 10. C. gloeosporioides was also found to grow and sporulate well between pH 4.0 and 6.5. Best growth was obtained at pH 6.0 (39). Harder (18) found that optimum growth of the oat isolate of C. graminicolum occurred at pH 7.5.

The purpose of this investigation was to determine whether the 6 isolates of C. graminicolum differed in their temperature and pH requirements for optimum growth.

Materials and Methods

(a) Effect of Temperature on Growth

Growth rate of 6 isolates of C. graminicolum was studied in a series of Petri dishes, each containing 20 ml. of potato-sucrose agar.

Inoculum was obtained by removing a 4 mm. disc of mycelium from the edge of an 8-day old colony. The disc was aseptically transferred to the centre of the medium in each plate. The fungus was then incubated for 7 days at temperatures of 10, 15, 20, 25, 30 and 35° C. Each treatment was replicated 4 times.

The diameter of each colony was measured when any isolate making the most rapid growth was nearing the edge of the medium.

(b) Effect of pH of Medium on Growth

The effect of pH of the medium on the growth of 6 isolates of C. graminicolum was determined by measuring the diameter of each colony produced in Petri plates containing 20 ml. of potato-sucrose agar.

The pH of each medium was adjusted by additions of HCl or KOH as required. A pH range of 3 to 11 was thus established, and growth was measured on media at one pH unit apart.

Preparation and transfer of inoculum were similar to those described under the section dealing with effects of temperature. The diameters of colonies were measured when the most rapidly growing culture had almost reached the edge of the agar medium.

Results

(a) Effect of Temperature

Fair to good growth of all isolates occurred at a temperature range of 15 to 35° C, with the optimum growth occurring between 25 and 30° C for all isolates except the one from oats with the optimum being at 20 to 25° C (Fig. 5).

At the optimum temperatures the rate of growth varied considerably, with the oat isolate making the slowest progress and the reedgrass isolate the most rapid growth. Isolates from reedgrass and wild oats had the highest temperature of 30° C for maximum growth, but they grew well over a 25 to 30° C

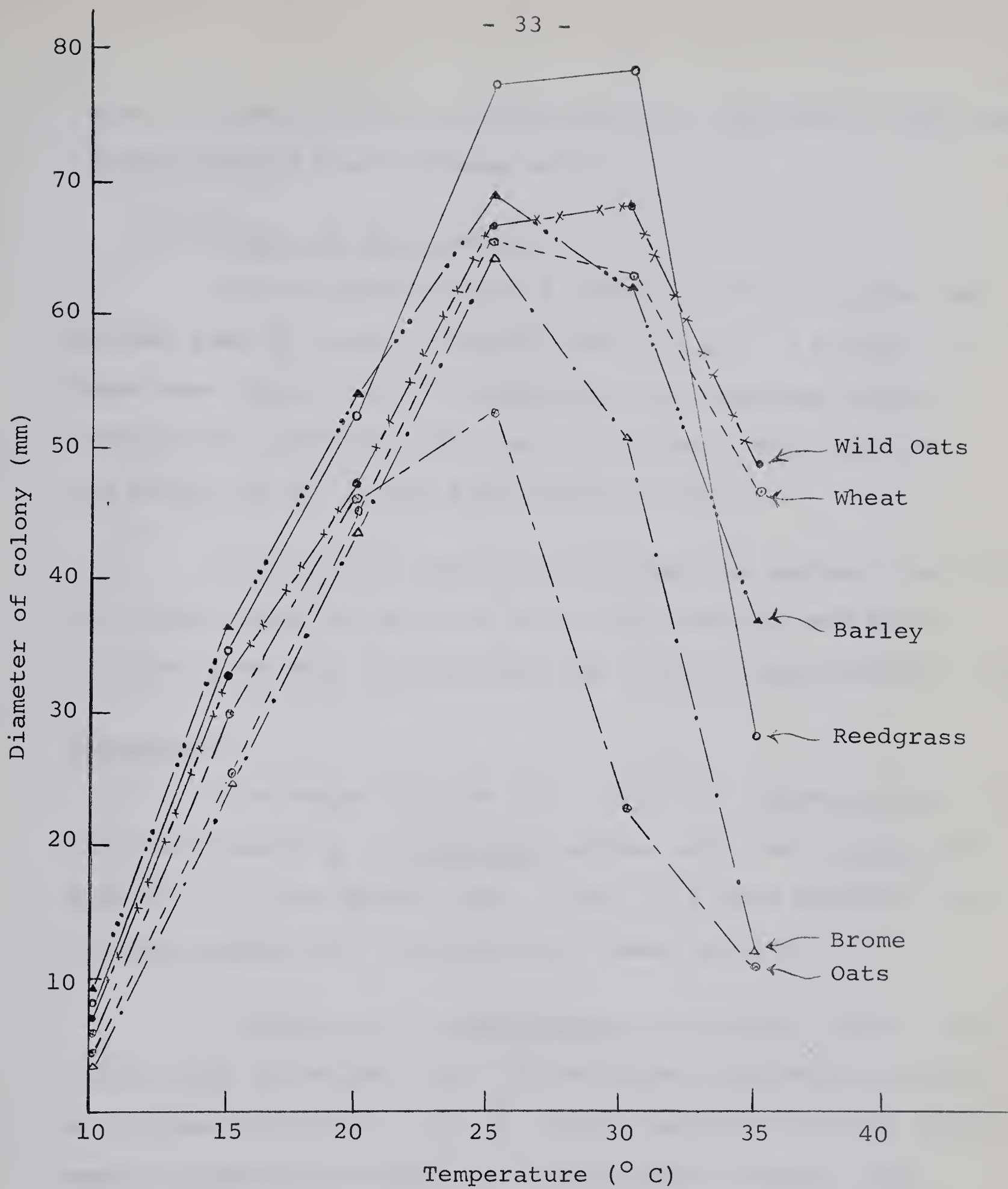


Fig. 5. Diameters of colonies of isolates of Colletotrichum graminicolum obtained on potato-sucrose agar at different temperatures.

range. A sharp drop in the rate of growth occurred in all other isolates beyond their optimum point.

(b) Effect of pH of Medium

All isolates grew at a range of pH 3 to 11 but the maximum rate of growth occurred mostly at pH 5 - 8 (Fig. 6). There were considerable differences in the maximum rates of growth among isolates, with the oats colony reaching 82 mm. and barley 44 mm. in the same period of time.

The wild oat isolate maintained its maximum growth at the widest range of pH 4 - 8 while the reedgrass and barley isolates grew well in a narrower pH range of approximately 6 to 8.

Discussion

The temperature of 25 to 30° C for optimum growth of most isolates of C. graminicolum agrees with the findings of Edgerton (15) and Harder (18). There is little evidence for 2 optimum temperatures for growth of these isolates.

Isolates of C. graminicolum tolerated a fairly wide range of pH of medium, but the best growth was restricted to a narrower pH range of 5 to 8. This reaction to the pH of the medium corresponds closely to the findings of Small (35), Chowdhury (12), Tandon (37) and Harder (18).

The extent of variability among isolates of C. graminicolum in response to temperature and pH is not great among some isolates but reaches a considerable amount among a few of them.

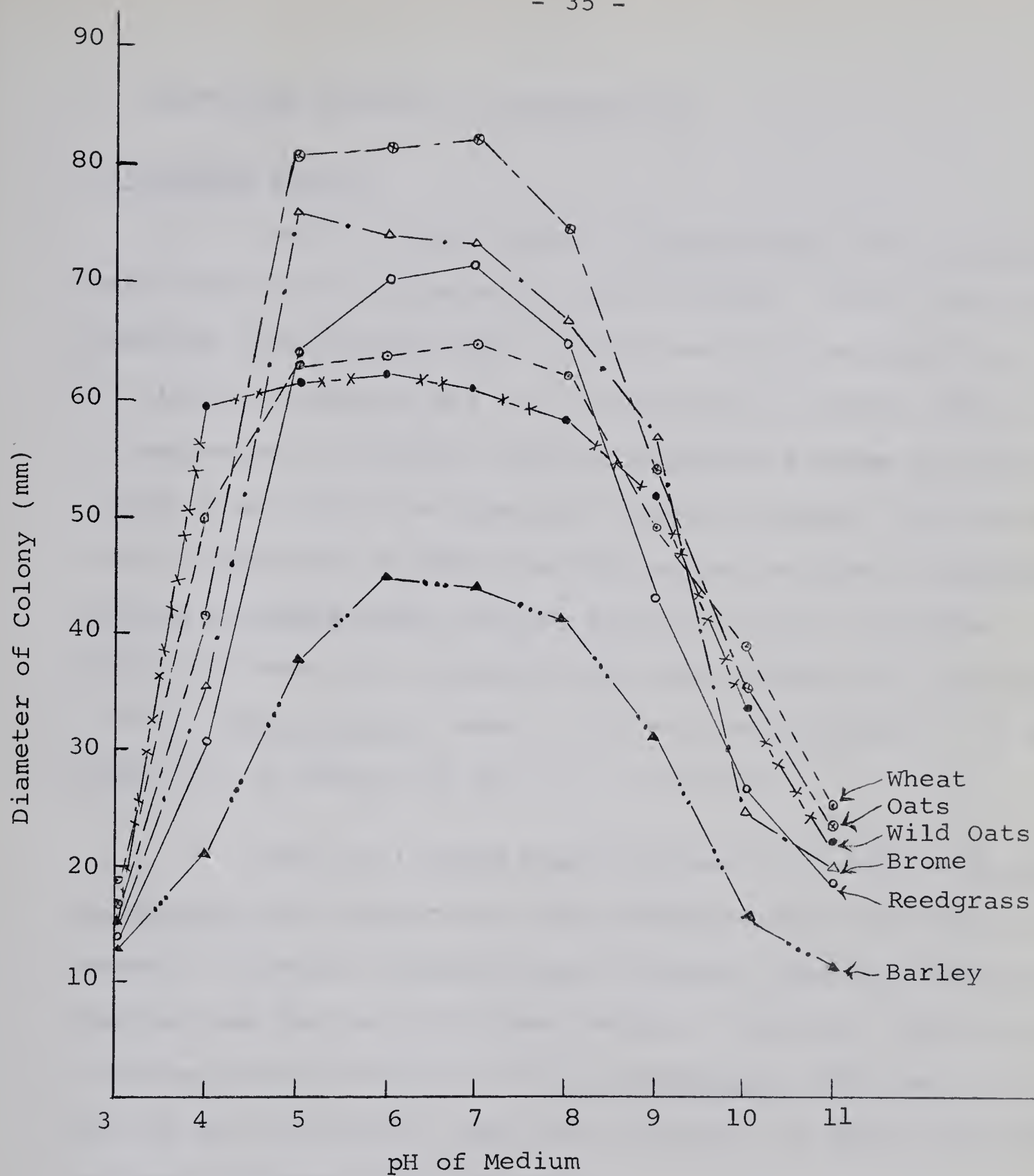


Fig. 6. Diameter of colonies of isolates of Colletotrichum graminicolum obtained on potato-sucrose agar at different pH levels.

2. Spore Size and Rate of Germination

Literature Review

Conidia of the forms of Gloeosporium and Colletotrichum were shown to be variable in size and shape. Shear and Wood (33) observed that conidia from a single acervulus varied from 10 to 25 microns in length and 3.5 to 6 microns in width. This range of variation in a single culture covered the range of variations found in all the forms they had studied. Burger (10) found a great difference in spore size of several strains of Colletotrichum gloeosporioides ranging from 11.5 to 20.3 microns. Selby and Manns (32) reported that the conidia of C. cereale (now C. graminicolum) were 18 - 26 microns long and 3 - 4 microns wide with an average of 22 x 3 - 5 microns.

Leach (21) found that the size of conidia of C. lindemuthianum was influenced by the medium on which they were produced. Different biologic forms, however, were not influenced to the same degree by the same medium. Extensive work on spore size was done by Bell (7) on C. graminicolum, and large variations were observed. This large variation in spore size occurred on many different kinds of media.

Germination is the initial stage in the development of mycelium from a spore. The ability of spores to utilize their stored reserves for metabolism enables them to germinate in water or minimal media (16). However, germination of spores, like any physiological process, is affected by environmental as well as hereditary factors.

The time required for germination varies greatly, even when mature spores are tested and other factors are kept constant. Gottlieb (16) showed that in Sclerotinia fructicola, as long as 10 hours can intervene between the time the first and the last spore germinate.

In this investigation conidia were measured as they appeared under natural conditions and also as they appeared in artificial culture. The period of time required for conidia to germinate was also observed for various isolates of C. graminicolum. These characteristics could provide further evidence of variability among isolates of the anthracnose fungus.

Materials and Methods

(a) Spore Size

Ten-day old cultures of the isolates of C. graminicolum grown on potato-sucrose agar were brushed gently to dislodge conidia in water. A drop of the conidial suspension was transferred to a microscope slide, covered with a cover slip and examined under a microscope. Measurement was made with a calibrated ocular micrometer. The lengths of 20 conidia were measured.

Conidia from diseased straw from the various hosts were also measured. Bits of straw containing acervuli of C. graminicolum were washed in tap water and placed in Petri dishes with wet filter paper lining the bottom and lid. In this moist chamber conidia were produced abundantly after

2 - 4 days. The lengths of 20 conidia were measured in the same manner as described for artificial cultures.

(b) Spore Germination

Sterile microscope slides were dipped into hot potato-sucrose agar and placed on a V-shaped glass rod in a Petri dish lined with wet filter paper. When the agar on slides hardened, a dilute conidial suspension from 10-day old slants were pipetted on the slide. Excess water was drained off.

The first observation for evidence of germination was made 2 hours after seeding the slide, and followed by observations at 30-minute intervals. The periods of time after which the first conidium germinated, and when the majority of conidia germinated were recorded.

Results

(a) Spore Size

The lengths of conidia varied considerably within each isolate regardless of whether they were produced in artificial culture (Table 3), or naturally on infected straw (Table 4).

The conidial length of the isolates appears to fall into 2 groups, i.e., those within the 16 - 28 micron range and those within the 10 - 22 micron range.

Table 3. Lengths of conidia of 5 isolates of Colletotrichum
graminicolum produced in artificial culture

Conidial Number	Length (microns) of conidia from				
	Wheat	Barley	Oats	Wild Oats	Reedgrass
1	16.2	11.1	16.2	16.2	12.1
2	16.2	12.1	16.2	16.2	14.1
3	16.2	12.1	16.2	17.2	14.1
4	18.2	12.1	16.2	18.2	14.1
5	18.2	13.1	16.2	18.2	14.1
6	18.2	14.1	17.2	18.2	14.1
7	18.2	14.1	18.2	18.2	12.1
8	18.2	14.1	19.2	18.2	16.2
9	20.2	14.1	20.2	18.2	16.2
10	20.2	15.2	20.2	19.2	16.2
11	20.2	16.2	21.2	19.2	16.2
12	21.2	16.2	22.2	19.2	16.2
13	22.2	16.2	22.2	20.2	16.2
14	22.2	18.2	22.2	22.2	19.2
15	22.2	18.2	24.2	22.2	19.2
16	24.2	18.2	22.2	22.2	20.2
17	26.3	18.2	22.2	22.2	20.2
18	26.3	18.2	25.3	22.2	20.2
19	28.3	15.2	26.3	23.2	20.2
20	17.2	16.2	27.3	24.2	22.2

Table 4. Lengths of conidia of 5 isolates of Colletotrichum
graminicolum produced under natural conitions on straw

Conidial Number	Length (microns) of conidia from				
	Wheat	Barley	Oats	Wild Oats	Reedgrass
1	22.2	18.2	18.2	22.2	20.2
2	30.3	22.2	20.2	20.2	28.3
3	22.2	18.2	16.2	22.2	26.3
4	28.3	16.2	18.2	22.2	28.3
5	22.2	20.2	19.2	20.2	22.2
6	24.2	20.2	18.2	22.2	22.2
7	18.2	20.2	20.2	26.2	26.3
8	24.2	14.1	18.2	22.2	21.2
9	22.2	18.2	18.2	20.2	24.2
10	24.2	17.2	18.2	24.2	24.2
11	20.2	16.2	20.2	22.2	24.2
12	24.2	13.1	20.2	24.2	28.3
13	24.2	14.1	20.2	20.2	24.2
14	20.2	14.1	19.2	20.2	26.3
15	18.2	20.2	18.2	24.2	24.2
16	22.2	20.2	21.2	24.2	24.2
17	20.2	10.1	22.2	22.2	22.2
18	24.2	12.1	18.2	20.2	18.2
19	18.2	18.2	20.2	21.2	16.2
20	22.2	20.2	19.2	22.2	20.2

An analysis of variance by the F-test, however, showed that there was no significant difference in spore size both within and between cultures.

(b) Spore Germination

Conidia from all isolates germinated readily within approximately the same time. Maximum germination occurred 5 - 6 hours after the conidia were sown. The shortest period of time for the first spore to germinate was 3 hours.

Discussion

The large variation in conidial length of isolates of C. graminicolum confirms the observations of other investigators (33, 32, 7). When only average lengths of conidia are compared it appears that the differences are significant. However, the large variations in size occurring within any one isolate are sufficiently great to rule out any significant differences as determined by the F-test.

The large variation in conidial size within any isolate shows that these organisms cannot be satisfactorily separated on this basis.

The period of time required for conidia to germinate varied considerably over the 5 - 6 hour interval. There was no difference in spore germination between isolates, and therefore, this property cannot be used as a criterion for distinguishing these isolates of C. graminicolum.

3. Colony Characteristics

Literature Review

Variations in colony characteristics have been observed in species of Colletotrichum. Bell (7) obtained 2 types of cultural characteristics of Colletotrichum. Those from the cereals, wheat, rye, oats and barley produced aerial dark gray mycelium, nearly smooth, about 1 mm. thick, with large numbers of spores. He referred to these as the dark or typical type. The other group, the light type collected from Dactylis glomerata, had a scant, nearly white, aerial mycelium. Isolates from Poa pratensis were practically colorless in culture with little aerial mycelium. He also obtained different cultural variants by isolating single spores from overgrowths appearing in the culture.

The phenomenon of variants arising in cultures of fungi is common especially in Fungi Imperfecti. Hansen (17) attributed these changes to the 'dual phenomena' of fungi, meaning that they are composed of 2 culturally distinct individuals. The mechanisms of variation include mutation, anastomosis of hyphae or hybridization.

Chona and Hingorani (11) showed that mutation occurred in Colletotrichum falcatum, and that richness of medium and age of culture are conducive to the occurrence of such mutations. The frequency of mutations increased with the age of cultures.

Materials and Methods

Twenty ml. of potato-sucrose agar were added to Petri dishes. Three single spores were isolated from each culture and seeded in each plate. The material was incubated at room temperature for 7 days.

Results

The colony characteristics were more or less distinct for each isolate and there was no obvious difference among colonies arising from sister spores (Fig. 7).

These colony characteristics may be briefly described as follows;

- A. Wheat isolate - mycelium dark to olivaceous green, fluffy, hyphal tips creamy white.
- B. Oats isolate - white or cream color, slightly raised at centre but receding towards edges.
- C. Barley isolate - colony white with gray patches, white overgrowths, ridged.
- D. Brome isolate - uniformly greyish white.
- E. Wild oats isolate - gray to black in the centre with wide margin of white, centre depressed.
- F. Reedgrass - dark grey in middle with lighter gray at margins, centre depressed.

Discussion

All colonies when first isolated were darker in color and resembled the wheat isolate. But subsequent subculturing on potato-sucrose agar has resulted in striking changes. The

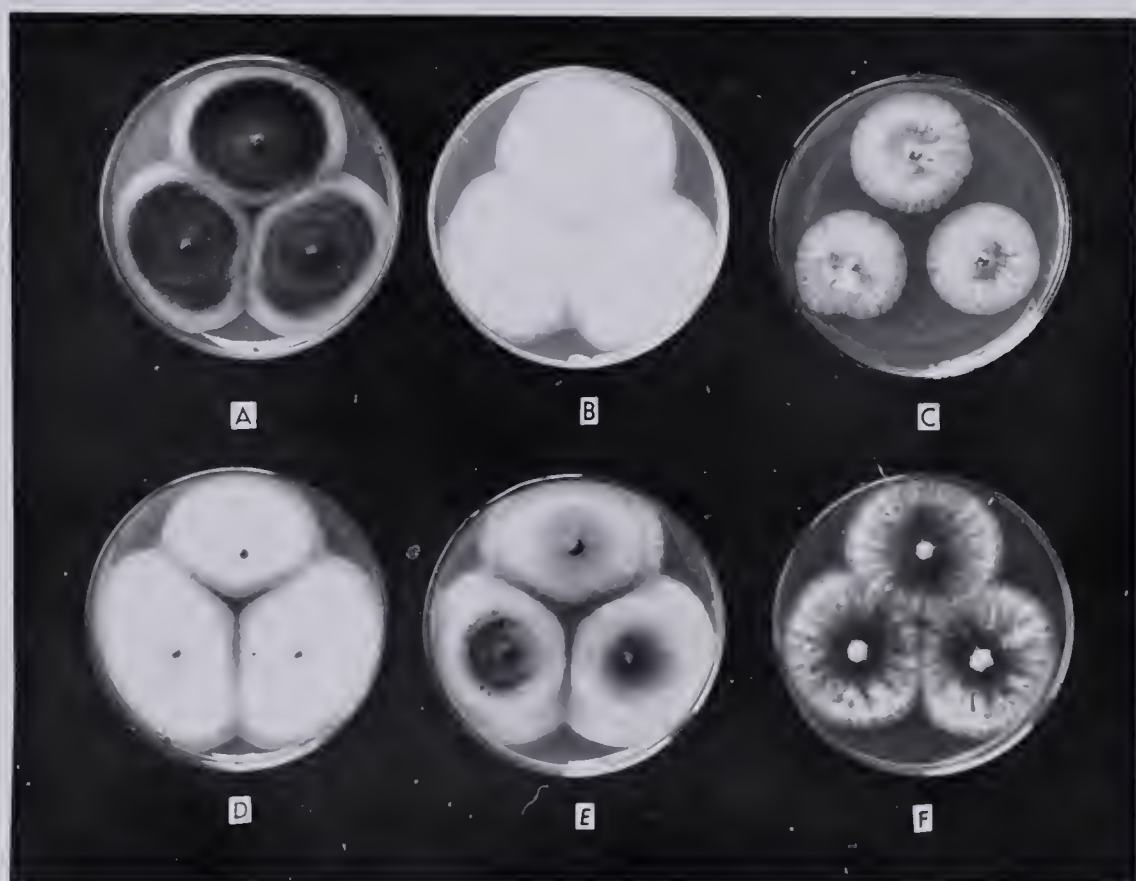


Fig. 7. Colony characteristics of 6 isolates of *Colletotrichum graminicolum* (A) Wheat, (B) Oats, (C) Barley, (D) Brome, (E) Wild Oats, (F) Reedgrass.

rate of growth was also reduced from that of the wild type. Wheat and wild oats retained their original colony characteristics most closely.

4. Numbers of Nuclei

Literature Review

There appear to be no published reports on the nuclear phenomena of hyphae of C. graminicolum or related species.

Since extensive variability occurs in some characteristics of the isolates of this fungus, it was considered necessary to describe the nuclear phenomena in an attempt to show further variability and thus to explain possible reasons for these variabilities.

Materials and Methods

In preparation for staining of nuclei, each isolate was seeded in a water suspension on an agar-covered microscope slide. The seeded slide was placed on a V-shaped glass rod in a Petri dish lined with moist filter paper and incubated for 2 - 3 days. In this period of time the fungus grew well but not too thickly to interfere with staining procedures.

The slides were removed from Petri dishes and air-dried for approximately 1 day. They were then stained by the HCl-Giemsa method as described by Robinow (29).

Results

Hyphal cells of isolates from barley (Fig. 8), wild oats, oats and reedgrass are multinucleate, while those from wheat (Fig. 9) and brome are uninucleate.

In all multinucleate hyphae the hyphal tips contained 5 to 17 nuclei per cell while hyphae about 4 to 5 cells back of the tip contained mostly 3 nuclei per cell (Fig. 10), although larger numbers were observed occasionally. In wheat (Fig. 11) and brome isolates cells back of the hyphal tip were also uninucleate.



Fig. 8. A hyphal tip cell of the barley isolate of Colletotrichum graminicolum showing its multinucleate condition.



Fig. 9. A hyphal tip cell of the wheat isolate of Colletotrichum graminicolum showing its uninucleate condition.

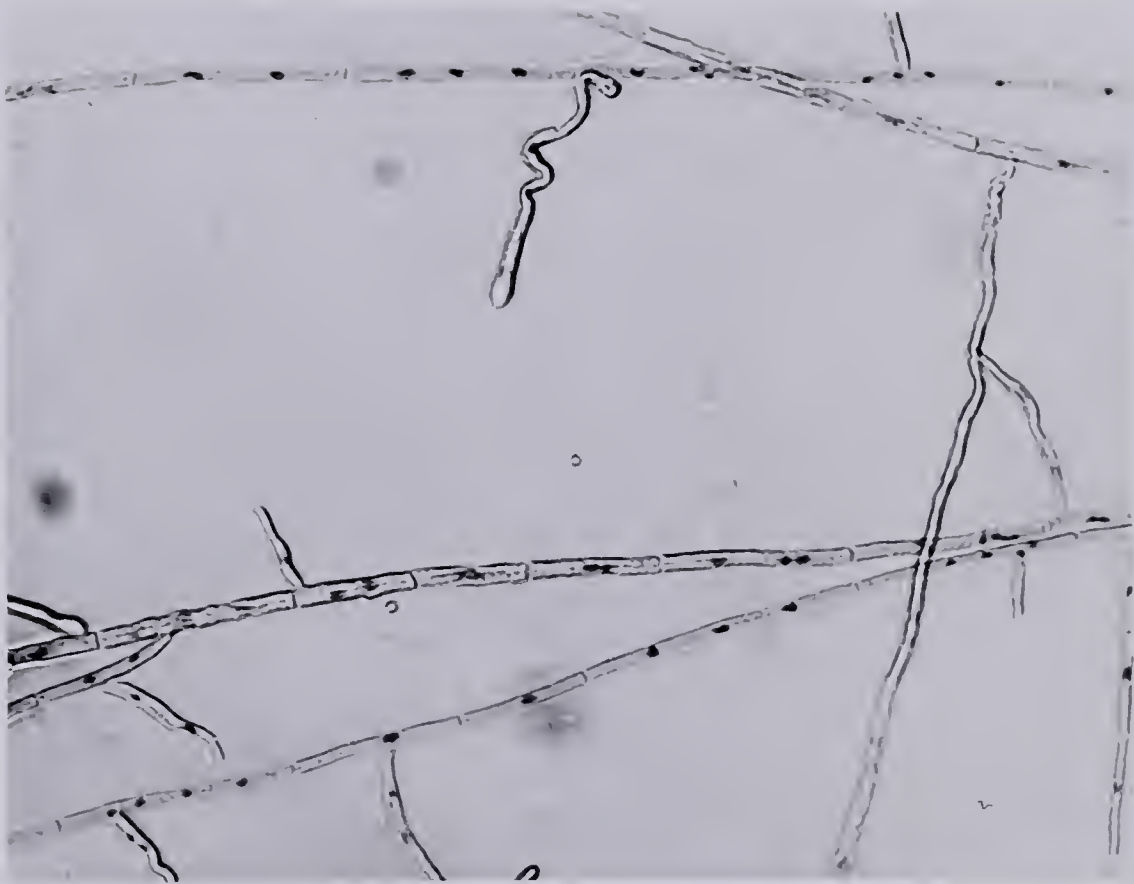


Fig. 10. Hyphal cells of barley isolate of Colletotrichum graminicolum a few cells back of the tip showing mostly 3 nuclei per cell.

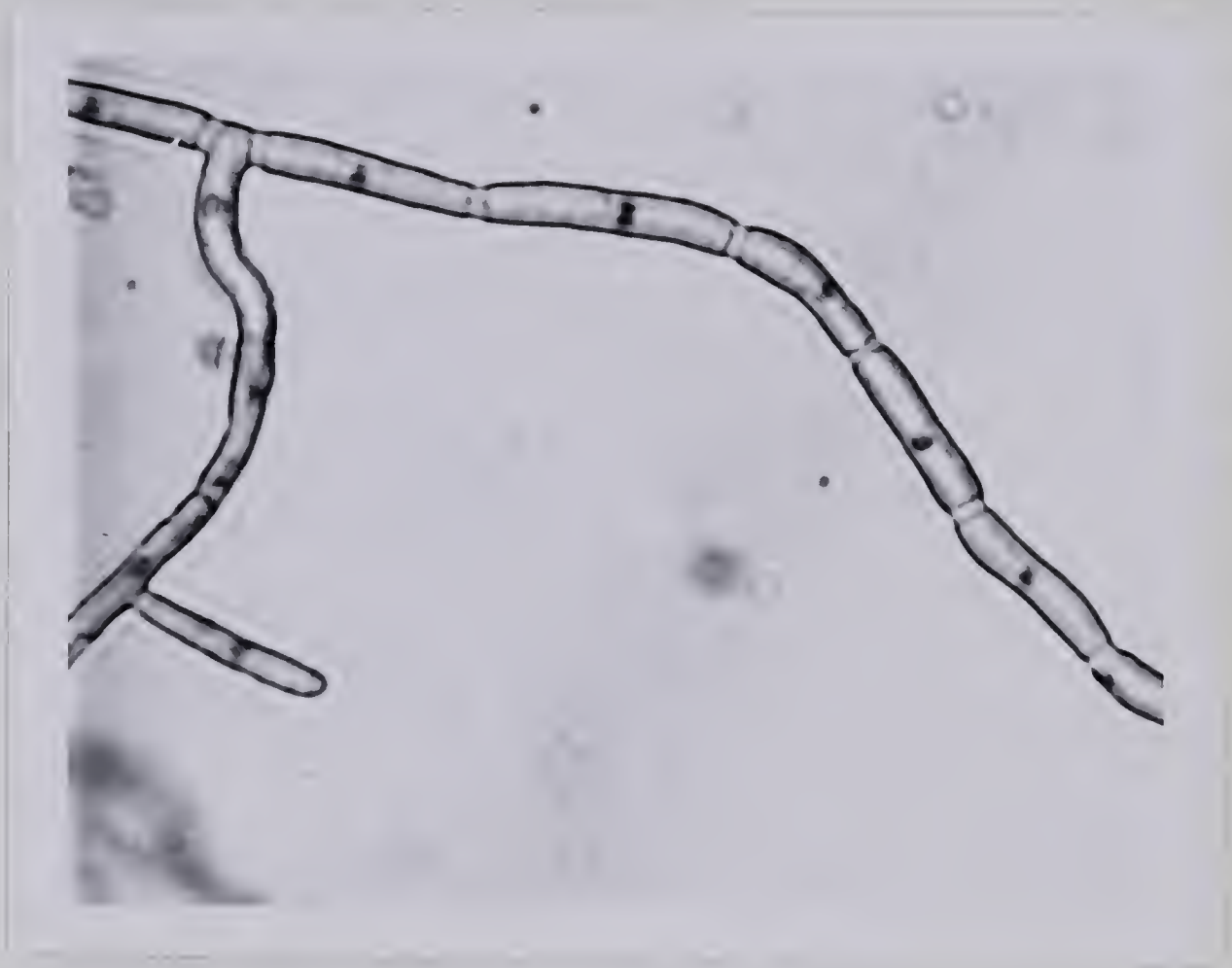


Fig. 11. Hyphal cells of the wheat isolate of Colletotrichum graminicolum a few cells back of the tip showing their uninucleate condition.

GENERAL DISCUSSION AND CONCLUSIONS

A high degree of variability occurs in physiologic properties and morphologic features among the 6 grass isolates of C. graminicolum. Perhaps the most striking variability among isolates was found in their growth response to inorganic and organic sources of nitrogen. Ammonium nitrate was the best source but there were great differences among isolates in the amount of growth attained on this medium. When grown on potassium nitrate the differences in maximum amount of mycelium produced were somewhat lower for each isolate, but the degree of variability among isolates was equally as great. Ammonium sulfate, as a source of nitrogen, differed from the other two compounds in that the amount of growth was somewhat less in most isolates and the differences between them were accordingly reduced. This is illustrated by the observation that in ammonium nitrate the difference in weight between the largest and the lowest was 400 mg. while in ammonium sulfate this difference was only 55 mg. The drastic lowering of pH in the ammonium sulfate medium may have been responsible for inhibiting the full potential of this compound for the growth of the isolates.

A high degree of variability also occurred on asparagine. Only the cereal isolates were compared here, but the difference between the maximum and minimum amount of growth was approximately 390 mg. A somewhat similar large difference in weight obtained in two isolates of Helminthosporium gramineum on asparagine was shown by Skoropad and Arny (34).

Variability in the pathogenicity of the grass isolates on 4 cereals was also demonstrated. The brome isolate did not infect any of these plants but was highly pathogenic on brome.

Comparison of some growth or reproductive characteristics showed large and minor differences. Temperatures at which the isolates produced maximum growth were similar for all isolates except the one from oats. It produced its best growth at about 5° C lower than the other isolates. Harder (18) observed that the oat crop is most severely affected by the anthracnose disease in Alberta. The greater susceptibility of oats may be partly caused by lower temperatures that usually occur in Alberta and favor the growth of this fungus.

The wide range in pH which allows maximum growth of all isolates indicates that this factor is of no consequence in prevalence and severity of anthracnose in Alberta.

Both spore size and rate of germination were extremely variable within isolates and, therefore, they cannot be considered as important in distinguishing them.

Nuclear phenomena of isolates present an interesting difference in that 2 isolates are uninucleate and the other 4 are multinucleate. These differences in nuclear numbers do not appear to be correlated with any other isolate characteristics observed in this investigation.

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